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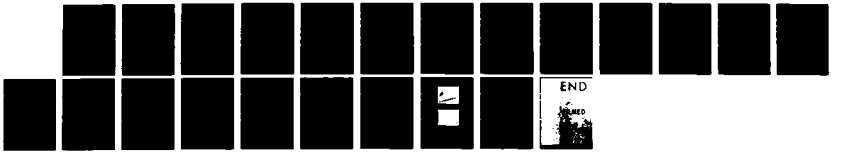

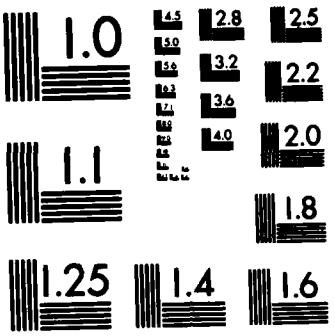

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Report Number 1

DEVELOPMENT OF MONOCLONAL ANTIBODIES TO T. B. RHODESIENSE ANTIGENS

Annual Report

Gary H. Campbell, Ph.D.

and

Janis Giorgi, Ph.D.

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20 Abstract -

This report, covering work performed from 1 September 1979 - 15 January 1980, describes methodology of tissue culture, hybridization procedures, immunization schedules and antibody assays developed to produce monoclonal antibodies to variant specific surface antigens of Trypanosoma b. rhodesiense organisims of the Walter Reed Army Trypanozoon antigenic type (WRATat) serodeme. Monoclonal antibodies have been produced to WRATat 3 and 5,

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-20 Abstract -

A laboratory has been developed for the ongoing production of monoclonal antibody reagents to Trypanosomes. These reagents will be critical in assessment of antigenic variation and in potential vaccine development programs.

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SUMMARY

Specific antibody reagents are necessary for studies of the biological nature of antigenic variation in African trypanosomes. Several clones of Trypanosoma b. rhodesiense organisms with different variable antigen types (VATs) have been isolated and constitute the Walter Reed Army Trypanozoon antigenic type (WRATat) serodeme. The objective of this work is to test various parameters of the "hybridoma" system (cell type, antigen preparation, hybridization procedures, assay systems, etc.) to determine the conditions which will allow production of monoclonal antibodies to large numbers of VATs with the greatest conservation of resources. Fusion of P3 X63 Ag8 plasmacytoma cells with spleen cells immunized with trypanosomal antigens has been successfully accomplished with the resulting production of monoclonal antibody (Mab) to the variant specific surface antigen of WRATats 3 and 5. Assessment of Mab production has included the following assays: Enzyme-Linked Immunosorbent Assay (ELISA), Dried slide indirect fluorescent antibody assay (DIFA), Wet mount indirect fluorescent antibody assay (WIFA) and the fluorescence activated cell sorter (FACS). The DIFA was determined to be preferable in terms of primary screening for variant specific antibodies. The FACS assay is under development as a secondary, more analytical tool for quantitative assessment of variant specific surface reactivity. The use of the combined methodologies has resulted in a working laboratory for the production of monoclonal antibodies against antigens of T. b. rhodesiense.

FORWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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BODY OF THE REPORT

I. Introduction

Trypanosomiasis of both man and cattle is one of the major health problems of the African continent. An obvious solution to this problem is the development of a vaccine. Even though trypanosomes elicit a strong antibody response in experimental animals, efforts at vaccine development have been hampered by the ability of trypanosomes to alter their surface coat characteristics and become resistant to the previously induced immune response. Recent collections of clones of organisms with antigenically distinct surface characteristics have allowed a starting point for the study of antigenic variation (1). Because of the extreme variability of the organisms, resulting in minor contamination of the clones, the production of antibody reagents with unquestionable specificity to surface coat antigens has been difficult. A recent technology has been developed which may offer a solution to the problem of antibody specificity and aid the study of the biological characteristics of antigenic variation in trypanosomes. This is the development of monoclonal antibodies through cell hybridization procedures involving fusion of mouse myeloma cells to spleen cells from specifically immunized animals. These procedures provide the ability to obtain continuous cell lines making monoclonal antibodies that are specific for a given single antigenic determinant.

The purpose of the present work is to determine the feasibility of producing monoclonal antibodies against Trypanosoma b. rhodesiense organisms of differing variable antigen types (VATs). The approach is to define various parameters of the "hybridoma" system (cell type, antigen preparation, timing after immunization, assay systems, etc.) which will allow the successful production of monoclonal antibodies to as many antigenic types of the Walter Reed Army Trypanozooan antigenic type (WRATat) (1) serodeme as possible.

This report covers work performed on this project from the starting date of 1 September 1979 until 15 January 1980. It includes the collaborative efforts of the principal investigator, Gary H. Campbell, co-investigators Janis Giorgi and George Cain (on Sabbatical leave from University of Iowa), consultant Noel Warner, and technicians Susan Griswold and Vickie Loemker. Generous consultation was also obtained from Klaus M. Esser of WRAIR. The report will describe various methods and techniques that have been used to arrive at a feasible procedure for production of monoclonal antibodies

against many VATs of T. b. rhodesiense.

II. Methods

This section will describe the major methodology and techniques that have been used or are in progress in this work.

A. Immunization procedures. The organisms used for these studies were derived from clones of organisms comprising the WRATat serodeme of T. b. rhodesiense (1). All organisms are stored as stabilates in 10% glycerol at -70°C, and after thawing, the organisms are grown for 4 days in irradiated (750R) C57B1/6J mice before injection into non-irradiated mice to establish an infection, or before further passage in irradiated mice for harvest of organisms to use as antigen. Organisms are isolated from infected blood by passage of the blood over DEAE 52 columns (2). The organisms are washed and adjusted to 5×10^8/ml in a phosphate buffer containing 5×10^{-5}M 2ME and 5×10^{-3}M HEPES, pH 5.6. Incubation of organisms at 0 C for 18 hours releases some of the surface antigen while leaving many of the remaining organisms intact. SDS-polyacrylamide gel electrophoresis (3) reveals at least 15 protein components in the preparation, although an enrichment of the surface coat glycoprotein is evident. This surface eluate antigen has been used as a second immunization or boosting antigen at a protein concentration of 0.1mg/mouse, injected intraperitoneally (i.p.) or intravenously (i.v.) three days before cell fusion experiments.

An alternate antigen to the surface eluate antigen is simply a freeze-thaw antigen prepared from DEAE isolated organisms. A pellet of organisms is diluted 1:4 with distilled water and repeatedly frozen at -20°C and thawed. Injection of 0.1 ml of this suspension is roughly equivalent to injection of 2×10^8 organisms or about 0.9 mg protein.

Two basic procedures of immunization, either primary or secondary, have been used to obtain spleen cells for fusion experiments. For primary immunization, mice are infected with 1×10^6 - 7×10^6 organisms of the appropriate VAT and cured on day 7 with 0.5mg Berenil. Cell fusions are performed three days later. For secondary immunization, mice that have been infected and cured are boosted from 1-3 months later with surface eluate or with freeze-thawed organisms, and cell fusions are performed 3 days later. The VATs that have been used or are being used in current experiments are WRATats 3, 5, 8, 9, and 12. Other VATs are being grown from reference stabilates for future use.

B. Hybridization procedures. The procedures which we have adopted for developing hybridoma cell lines forming monospecific antibodies against trypanosomes are those which have been successfully used in many other laboratories for producing hybridomas against a variety of antigens (4). Several aspects of our particular cell fusion technology are discussed below.

1. Cell lines used for fusions: Several tumor cell lines are now available in our laboratory for use in fusion experiments. They are listed in Table I. The P3 X63 Ag8 cell line, here termed X63, has been used for the majority of the fusion experiments. In addition, fusions have been attempted using a variant of X63, called P3-X63 Ag8.6.5.3, termed 8.6.5.3, which is a nonsynthesizer of immunoglobulin. These cell lines are maintained in Dulbecco's modified essential medium with 5 to 10% fetal calf serum (FCS) and are used for fusions when they are in the log phase of growth. All of these lines are deficient in hypoxanthine guanine phosphoribosyltransferase and, thus, do not grow in selective medium containing aminopterin and supplemented with hypoxanthine and thymidine. In contrast, tumor cells which have fused with spleen cells from immunized animals will grow in this selective medium. Non-fused spleen cells will not grow.

2. Fusion techniques: Two different fusion techniques have been attempted. One procedure, obtained from Klaus Esser at the WRAIR, has been used for fusions involving X63. The other, obtained from John Kearney who developed the 8.6.5.3 cell line at the University of Alabama has been used for fusions involving that cell line. The details of the fusion protocols are outlined below:

a. Fusions with X63: Spleen cells from an immunized mouse are removed into medium and a single cell suspension is prepared. RBCs are lysed with 0.17M NH_4Cl and the cells washed and resuspended in medium with no FCS. Next, 10^7 X63 cells and 10^8 spleen cells are put into a 50 ml conical centrifuge tube and washed once with medium lacking FCS. To the pellet of tumor/spleen cells, 0.2 ml of warmed (42°C) 30% PEG MW=1,000) is added, and the pellet is resuspended by tapping the tube. The cells are then centrifuged for 3 minutes at 500 x g, at room temperature, and after an additional 2 minutes, 5 ml of medium without FCS is added slowly. After an additional 2 minutes, the cells are resuspended, spun, and diluted to a final concentration of X63 cells of 3.33×10^5/ml (i.e., for 10^7 cells, 30 ml of medium. This medium, called enriched HT medium, contains

20% FCS, 10% NCTC (Microbiological Associates), 1% glutamine (Gibco), 1% Na pyruvate (Gibco) 1% hypoxanthine (10^{-4}M stock), 1% thymidine (1.6×10^{-5}M stock), and penicillin/streptomycin. The cells are then distributed into 96-well, flat bottomed microtiter plates, using about 0.05 ml/well. Thus, about 500-600 wells are prepared from one mouse spleen. After 24 hours, an equal volume of medium containing a 2X concentration of aminopterin (8×10^{-7}M) is added. The cells are fed (1-2 drops of medium is added) after 3 days, and thereafter, the medium is changed every 3 to 4 days using enriched HT medium until growth is seen. Growth is evident by examination of the contents of each well by phase contrast microscopy, and the medium turns acid in wells which have heavy growth. At this time, the medium is changed twice in one day, and the supernatant is sampled for antibody 24 to 72 hours after the second medium change. Thereafter, the medium is changed every two days until the cells are transferred or frozen.

b. Fusions with 8.6.5.3: These are performed with only minor variations from the above procedure, the major point being that PEG with a molecular weight of 4000 must be used, and the PEG is left on the cells for only 30-60 seconds, and then diluted out. Another variation is that after fusion, the cells are distributed into 24-well tissue culture plates in a volume of 1 ml of HAT medium.

3. Cloning of hybrids producing variant-specific antibodies: Cells in wells with positive supernatants are cloned by limiting dilution by plating small numbers of cells into wells which contain 10^6 fresh BALB/c thymocytes per well. Approximately 10, 3, or 1 cell per well on an average are plated since the plating efficiency of hybrids during early growth is often low. Because the Poisson distribution predicts that there will be 1 cell/well ($p<0.05$) if $\leq$37% of the cloned wells have growth, only those growing wells which appear under conditions which allowed growth in $\leq$37% of the wells are saved. Supernatants from these wells were tested for antibody activity, and cells from positive wells are grown up in 1 ml wells with feeder layers of thymocytes in HT enriched medium. Eventually, clones which are secreting antibodies of interest are grown up in larger quantities.

All supernatants from positive wells are frozen at -20°C throughout. Cells are frozen in liquid nitrogen at the time the original positive wells are cloned and again when positive clones are obtained.

C. Assay methods. In screening for the presence of trypanosome-specific antibody in hybridoma supernatants, we have employed four different assays. The use of two different assays per screening allowed for independent assessments of positive reactivity. Moreover, we have attempted to design each method so as to determine whether the antibodies produced are VAT-specific.

1. Dried Slide Indirect Fluorescence Assay (DIFA): Antigen slides containing trypanosomes for indirect fluorescent antibody tests were prepared in two ways:

a. Blood smears: Blood from mice infected with different WRATat's was mixed in known proportions, and blood smears were prepared, dried and stored frozen (-20°C) for up to six weeks.

b. DE smears: Trypanosomes of different WRATat's were separated from host blood on DEAE cellulose (2), washed in PBS (pH 8.0) containing glucose, suspended in a known volume of Fetal Calf Serum (FCS) and counted in a hemacytometer. Known volumes of two VATs were mixed and smears were made, each from 10 ul of the mixture containing a total of 3×10^5 organisms.

Frozen slides were immediately placed in a dessicator and thawed at room temperature for 30 minutes. When dry, the slides were subdivided into 10-14 compartments using fingernail polish. 20 ul of undiluted hybridoma supernatant were placed into each of these compartments and the slide was incubated for 20 minutes at 37°C in a moist chamber. The serum was then aspirated from each compartment, and the slide washed 3X for 5 minutes each in 0.1 M phosphate-buffered 0.15 M saline (PBS), pH 7.2. After washing, each slide was flooded with a mixture of fluorescein-conjugated goat anti-mouse IgM and IgG (7s globulins) (Meloy, Inc., Springfield, Va.), each diluted 1:10 in a conjugate diluent containing 2% Tween 80 and 0.02% Evans Blue dye in PBS. Slides were incubated in conjugate for 20 minutes at 37°C in a moist chamber, washed 3X for 5 minutes each in PBS, dried by blotting, and mounted in 10% PBS in glycerol. Each well was then examined with a Zeiss phase-contrast microscope equipped with epifluorescence, and positive fluorescence was determined by comparison with control compartments that had been incubated with non-VAT-specific mouse antiserum.

2. Wet-mount indirect Fluorescence Assay (WIFA): Trypanosomes separated from other blood components by DEAE

cellulose were washed 3X in PBS (pH 7.2) at 4°C and diluted to a known volume in the same buffer. They were then fixed by adding an equal volume of cold 0.2 M sodium cacodylate buffer (pH 7.2) containing 4% glutaraldehyde (Ted Pella, Inc., Tustin, California). Fixed trypanosomes were dispersed by mixing for 2-3 minutes on a vortex mixer and stored at 4°C for as long as 4 days.

For use in the WIFA, fixed trypanosomes were washed 5X in cold PBS by centrifugation at 550 X g to remove excess glutaraldehyde, and apportioned into 5 ml disposable glass test tubes. The cells were centrifuged again, the supernatants removed, and undiluted hybridoma supernatants were added to the tubes. Following a 15 minute incubation at 4°C, the cells were washed 3X by centrifugation and incubated for an additional 15 minutes in the conjugate, diluted as described above. Conjugate was then removed by 3 washings in PBS, and the trypanosomes were observed in the fluorescent microscope. Positive reactivity was typically noted as a ring of bright fluorescence at the perimeter of the cell.

3. Analysis by Flow Cytometry (FACS): Cells prepared for WIFA as described above were diluted to a concentration of 1×10^5/ml in PBS, filtered through fine nylon mesh and analyzed with a Becton-Dickinson fluorescence-activated cell sorter (FACS III). Reactivity was determined by comparison of the test sample with organisms treated with normal mouse serum or tumor cell supernatant.

4. Enzyme-linked Immunosorbent Assay (ELISA): Microtiter plate ELISA assays were carried out according to the methods of Voller et al., (5, 6) using as antigen either whole trypanosome freeze-thaw preparations (10-50 ug protein per well) or surface eluate antigen (5 ug per well), both prepared as described above. The primary antibody was undiluted hybridoma supernatant and the conjugate was peroxidase-linked goat anti-mouse IgG (obtained from Bionetics, Kensington, MD.). Positive reactivity was assessed qualitatively by examination of plates in a microtiter plate viewer after three hours; variant specificity was determined by repeating the assay against ELISA antigen prepared from organisms of a heterologous VAT.

III. Results

The results of 10 hybridoma fusion experiments are shown in Table II. Conditions, including immunization, tissue culture

and assay methodology, have been defined which have lead to the production of variant specific monoclonal antibodies against more than one antigenic type of the WRATat serodeme.

In four experiments using X63 cells and spleen cells from primary immunizations, many fusions were obtained; however, only 3 wells produced detectable antibody (Table II). In contrast, secondarily immunized spleen cells (experiments 7 and 9)yielded a total of 375 fusions, including 85 antibody producers. Nineteen of these were variant specific.

The limiting dilution method was used to establish clones of cells from 5 antibody producing wells in experiment 7. The number of clones obtained and the specificity of their antibody reactivity is shown in Table III. From 2-16 clones were obtained from each cloning attempt. From each attempt, at least one clone was obtained that showed reactivity of the same specificity as the parent population of cells.

In the fusion experiments described in Table II, two tumor cell lines were used. P3 X63 Ag8 cells were successfully used for fusion and subsequent antibody production. In two experiments using the P3 X63 Ag8.6.5.3 line, fusions were not obtained. Further experiments will be performed with this line using different lots of PEG as well as shorter exposure of the cells during the fusion process.

A comparative experiment with various tumor lines (Table I) is being performed with spleen cells from mice immunized in the same manner as fusion 9 (Table II). This is being done to see if a different tumor line will fuse more efficiently or will produce more antibody producing fusions.

Although we have not yet categorized the monoclonal antibodies produced as to heavy chain class, the screening assays used, especially the DIFA, did afford the opportunity to describe their reactivities. Thus, hybridoma supernatants assayed in the DIFA could be categorized as variant specific (VS) or non-variant specific (NVS). NVS supernatants could be further categorized as to their affinity for certain morphological features of the trypanosomes. In Fusion #9, e.g., fluorescence was confined to the flagellum in three different supernatants and in twenty, the fluorescence was confined to the posterior third of the organism. Similarly, eleven supernatants were nuclear specific; other non-variant specific reactions were not as easy to characterize, but ranged from patchy fluorescence to even, entire illumination of the cells. Even in the cases where the supernatants were clearly variant

specific, a small area on the posterior end at the position expected for the kinetoplast, showed brighter fluorescence than the remainder of the cell. Most likely, this is an optical effect due to folding-over of the cell membrane as it passes into the flagellar pocket. The DIFA was routinely used as the primary screening assay because, in addition to its advantages in assigning antibody reactivity of the hybridoma supernatants, it offered speed and convenience without undue losses in sensitivity. In initial screening attempts, the ELISA method proved roughly equal to the DIFA in sensitivity, as judged by standard comparisons with rabbit anti-WRATat antisera (1), and perhaps better in speed and convenience; however, it required quantities of supernatant too large to sacrifice in an initial screening, especially since it was necessary to run two microtiter plates simultaneously to assess variant specificity. The WIFA method, although somewhat more difficult to interpret, was slightly more sensitive in our hands than the DIFA, which was occasionally plagued by background fluorescence that resulted in loss of contrast.

Disadvantages to the WIFA as a primary screening assay however, offset its greater sensitivity. More organisms are required in the WIFA and they must be freshly fixed (cells fixed longer than 2-3 days begin to cross-link and lose their ability to bind the primary antibody). In addition, those supernatants containing non-variant specific reactivity to internal antigens cannot be determined; thus, an important by-product of the search for variant specific hybridomas would be lost. Also, preliminary evidence exists that some hybridomas to WRATat 1 may react better in the DIFA than in the WIFA.

By far, the most quantitative and sensitive assay in terms of surface reactivity that we have used, flow cytometry, has the disadvantage of being rather time-consuming and limited in the number of samples that can be processed. Despite these limitations, flow cytometry is an excellent "second stage" analytical tool for confirming results of other assays and further characterizing the reactivities of variant specific monoclonal antibodies.

Flow cytometry has been used previously to characterize trypanosomes (7) but, so far, it has not been applied to fluorescent antibody-stained organisms. An example of our use of this method in characterizing hybridoma supernatants 9-3-27 and 9-3-42 is illustrated in Fig. 1. The upper panel (A) shows a "dot plot", or simultaneous display of fluorescence intensity (ordinate) and cell size, as measured by light scattering

(abcissa). The sample analyzed contained WRATat 3 and WRATat 12, fixed separately in 2% glutaraldehyde and mixed in a 1:4 ratio (respectively) immediately before incubation in hybridoma supernatant 9-3-27. The higher fluorescence of the reactive (WRATat 3) trypanosomes is evident (a) as is a small (b), more highly fluorescent subpopulation which represents cells with a greater light scatter which are cross-linked in fixation. The nonreactive WRATat 12's (c) show varying degrees of cross-linking, producing a gradual linear increase in light scatter and fluorescent intensities; however, even the highest fluorescence intensity of these aggregates is less than that of the WRATat 3's.

Figure 1B shows the FACS assay of a pure population of WRATat 3 stained with normal tumor supernatant or each of 2 Mab to WRATat 3 (9-3-27 and 9-3-42). When the number of cells analyzed (ordinate) is plotted against fluorescence intensity (abcissa), the differing reactivities of the two hybridoma supernatants are evident, as is the very low fluorescence intensity of a control in which WRATat 3 organisms were incubated in supernatants from growing, unfused P3 X63 Ag8 cells. The shoulder peak in the control sample is due to cross-linking.

As more variant specific hybridomas are produced, we will continue to use flow cytometry in secondary level analysis of antibody specificity, and of quantitative reactivity.

D. Discussion. The results presented here demonstrate that in a period of approximately four months we have established a successful working laboratory for the production of monoclonal antibodies to individual variants of the WRATat serodeme. Experience has been gained with many of the variables of the project including immunization procedures, culture and hybridization techniques with different tumor lines, and different initial screening and analytical antibody assays.

The negative results obtained in early experiments using primary immunization may have been due to the lack of experience with screening assays as compared to later experiments. Klaus Esser at WRAIR has produced hybridomas against WRATat 1 with primary immunization. On the other hand, the parasitemia established after primary immunization may not have been sufficient to stimulate spleen cells to the state necessary for hybridization and antibody production. Studies with WRATats 3, 5, 9, and 12 have shown differential growth rates in initial parasitemia, with WRATat 3 producing the lowest parasitic load (Campbell, et al., unpublished).

Both experiments performed with spleen cells from mice immunized secondarily with surface coat antigen have produced monoclonal variant specific antibody. This is taken as an indication that monoclonal antibodies can be produced against all variants of the WRATat serodeme without the necessity of trying different immunization protocols for each VAT. This is an important consideration in attempting to produce antibodies against all the available VATs.

Comparative work with 2 hybridoma antibodies against WRATat 3 using the DIFA, WIFA and FACS has shown that the variant specific antibody is reacting to the outermost surface antigens of the intact cells. This is an important finding since some monoclonal antibodies against WRATat 1 may have little or no reactivity in a WIFA as compared to DIFA (Esser, personal communication). Reactivity to the exposed surface antigen would be important if the monoclonal antibody would be expected to have biological activity such as agglutination, lysis, or neutralization. The fluorescence activated cell sorter will be of use as an analytical tool to quantitate the surface reactivity of the variant specific monoclonal antibodies. It can also be used in various titration and blocking assays to determine which of the replicate antibodies (i.e., 3 vs. WRATat 3, 16 vs. WRATat 5) have identical specificity to exposed determinants on the organisms. This will be important in future characterization of the monoclonal antibodies.

The further development of the current and future hybridomas will be ongoing. Cells will be frozen in liquid nitrogen for future use. Ascites fluid will be prepared with cloned hybridomas to be used when larger amounts of the antibodies are needed. Frozen cells will be sent to WRAIR.

E. Conclusions. A successful working laboratory for the production of monoclonal antibodies to variant specific antigens of the WRATat serodeme of T. b. rhodesiense has been established. Variant specific monoclonal antibodies have been produced to WRATats 3 and 5. In all likelihood, monoclonal antibodies can be produced to all 16 of the VATS in the current collection of the WRATat serodeme.

TABLE I

TUMOR CELL LINES AVAILABLE AT UNM FOR USE IN FUSION EXPERIMENTS

Complete name	Short name	Parent line (strain)	Characteristics
1) P3 X63 Ag8	X63	MOPC-21 (BALB/c)	Plasmacytoma (λ, k)
2) P3-NSI-1-Ag4-1	NS-1 (Scripps)	"	Plasmacytoma (k, non-secreting)
3) P3-NSI-1-Ag4-1	NS-1 (Salk)	"	"
4) P3-X63 Ag8.6.5.3	8.6.5.3	"	Plasmacytoma (no Ig synthesis)
5) MPC11-X45-6TG	X45	MPC-11 (BALB/c)	Plasmacytoma (γ_2b, k)

TABLE II
SUMMARY OF RESULTS OF FUSION EXPERIMENTS

Fusion #	Immunization	Cell Line for Fusion	Growing Wells / Total Wells	Supernatant Assay Method	Total No. of Wells Producing Trypanosome-Specific Ab[a]	No. of wells with Variant Specific Reactivity
1	1^{o}WRATat 5[b]	X63[c]	134/750	WIFA	0	0
				ELISA	0	0
2	1^{o}WRATat 3	X63	75/920	WIFA	0	0
				ELISA	0	0
3	(discarded due to technical difficulties)					
4	1^{o}WRATat 8	X63	76/732	DIFA	3	0
				ELISA	2	0
5	1^{o}WRATat 5	X63	28/504	DIFA	0	0
				ELISA	0	0
6	1^{o}WRATat 5	8.6.5.3	no fusions	-	-	-
7	2^{o}WRATat 3 - SA[d]	X63	42/360	DIFA	6	3
				ELISA	0	0
8	2^{o}WRATat 3 - SA	8.6.5.3	no fusions	-	-	-
9	2^{o}WRATat 5 - SA	X63	333/912	DIFA	79	16
10	2^{o}WRATat 8 - FT	X63				

a. WIFA, wet-mount indirect fluorescence assay; DIFA, dried slide indirect fluorescence assay.

b. For primary (1^{o}) immunizations, mice were infected with 1-6.7 x 10^{6} organisms of the indicated WRATat and cured after 7 days with 0.5 mg Berenil. Hybridizations were performed 3 days after 1^{o} or 2^{o} immunizations.

c. Refer to Table I for further information on cell lines.

d. Secondary immunizations (2^{o}) were produced by boosting primary immunizations with either surface antigen (SA) or whole trypanosome freeze-thaw (FT) antigen (See methods section for antigen preparation) at 2-3 months after initial infection.

TABLE III
CLONING OF TRYPANOSOME-POSITIVE HYBRIDOMAS

Parent Hybridoma[a]	Parent Specificity	Number of Clones	DIFA Reactivity[b] VS	NVS	Neg.
7-3-10	VS	2	2	0	0
7-3-14	NVS	16	0	12	4
7-3-15	NVS	3	0	3	0
7-3-21	NVS	5	0	1	4
7-3-27	VS	6	2	0	4

a. Parent hybrids are designated as Fusion number-WRATat type-positive well number.

b. Supernatants reacting positively by DIFA were scored as to whether they were variant-specific (VS), non-variant-specific (NVS) or negative.

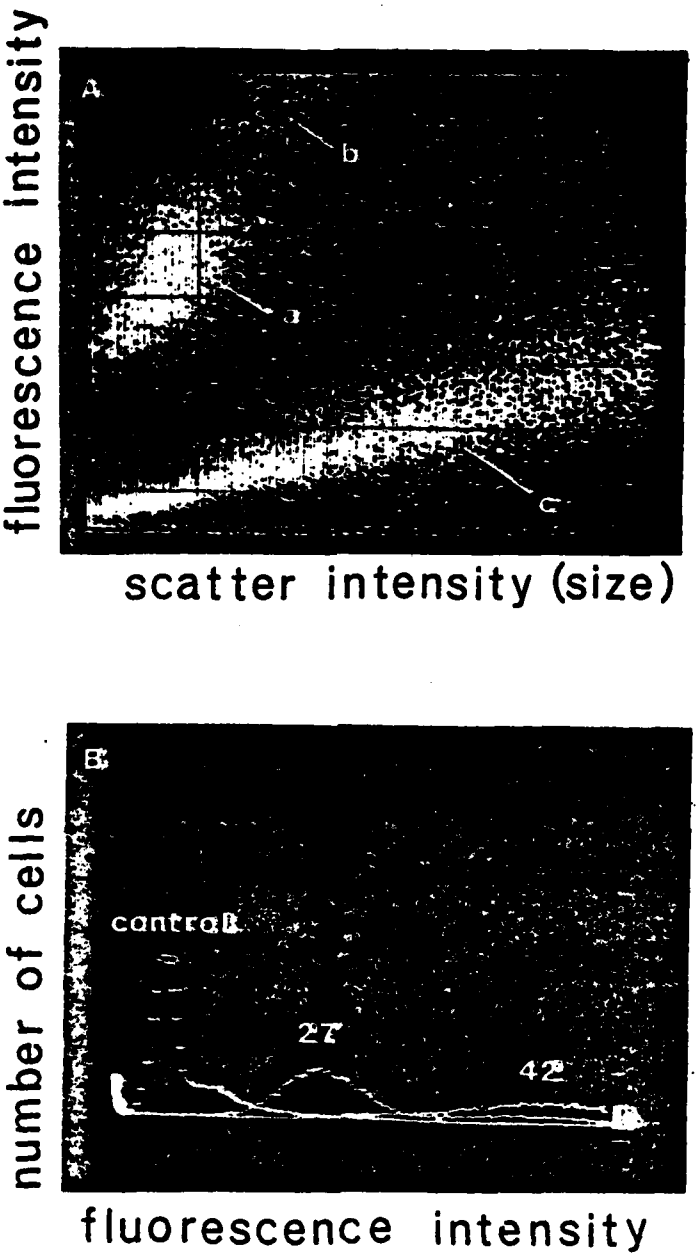

Figure 1: Analysis of anti-WRATat 3 hybridoma supernatants by flow cytometry: Dot plot (A) showing analysis of 1:4 mixture of WRATat 3 (a,b) and WRATat 12 (c); histogram (B) showing different distributions of fluorescence intensity for homogeneous populations of WRATat 3 incubated in hybridoma supernatants 9-3-27 and 9-3-42, and in control P3X tumor cell supernatant. The shoulder peak in the control analysis represents trypanosomes aggregated (cross-linked) in fixation. The FACS III was operated at 800V at a pre-amp gain setting of 8 X 0.5 and a fluorescence gain setting of 16 x 0.5.

LITERATURE CITED

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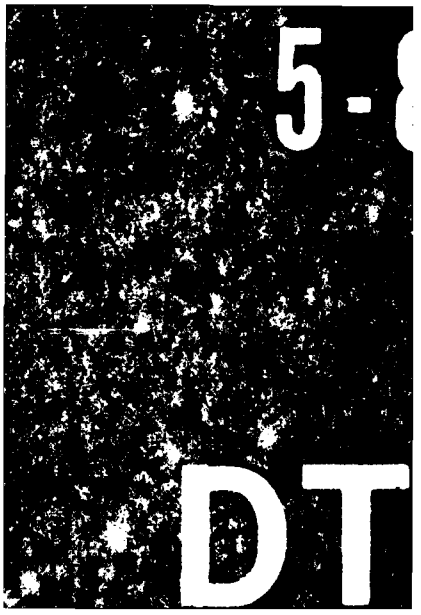
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